

# Mapping HIV-1 Gag interactions with inositol pentakisphosphate and nucleic acids

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## Objectives

## Structural Analysis of the Gag Assembly by Mass Spectrometric Protein Foot-Printing.

## Introduction

Gag polyproteins play important roles in the replication of HIV-1 viruses. Gag proteins assemble at fatty acid rich regions and form the spherical budding particles. The Gag proteins in these immature budding particles are cleaved by viral protease into proteins of the infectious virion. These cleavage events are accompanied by morphological changes resulting in the formation of the mature HIV-1 particle possessing a cone shaped core (Figure 1c). Since NMR or crystallographic analysis of the entire Gag structure was hampered by high flexibility of the polyprotein structures of individual Gag domains were determined.<sup>1</sup>

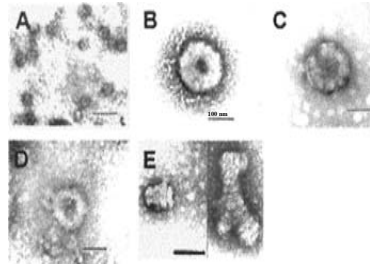


Fig. 1: Electron micrograph of negatively stained particles assembled in vitro of authentic, immature HIV-1 virion. Particles assembled in (A) no lysate, (B) 5% rabbit reticulocyte lysate, (C) Immature HIV-1 virion. Virus-like particles assembled in 2 μM of (D) IP5 (E) IP6.<sup>1</sup>

## Methods

In the protein foot-printing technique<sup>2</sup> (illustrated in Figure 3), the Gag protein (2.5 μM) was allowed to form complexes with inositol pentakisphosphate (IP5) and ssDNA (indicated concentrations, see Figures 5, 6 and 7) in 50 mM HEPES (pH 8.0), NaCl, 1 mM β-mercaptoethanol and 0.5 mM DTT. The complexes were subjected to lysine modification with 400 μM N-hydroxysuccinimidobiotin (NHS-Biotin). After incubation at 25°C for 30 min the reactions were quenched with 10 mM lysine. 2% SDS was added and heated for 70°C for 20 min. The mixture was treated with iodoacetamide (100 mM) for 45 min followed by quenching by 100 mM DTT. The protein samples were subjected to SDS-PAGE separation. Protein bands from the gel were excised and subjected to tryptic digestion. Finally the samples were analyzed by MALDI-ToF-MS (Kratos) and LC-MS/MS (CapLC-QToF II).

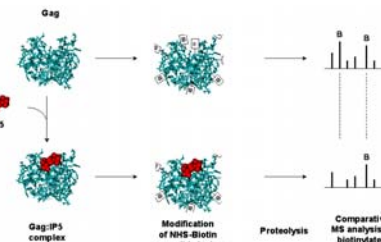


Fig. 3: Illustration of the mass spectrometric protein-footprinting by biotin labelling (B).

## Results

A detailed tryptic map of free Gag protein by MS was obtained. Assignment of tryptic fragments revealed greater than 80% amino acid sequence coverage of Gag (Figure 4). Biotinylated lysine peptides were identified from the mass spectra. (Table 1).

The IP5 binding sites were identified by using NHS-Biotin modification in the preformed Gag-IP5 complex. Among the 21 lysines readily modified on free Gag, only K30 and K32 were protected from modification in the Gag-IP5 complex (Figure 5). Inositol hexakisphosphate (IP6) did not support the Gag assembly (Figure 1), hence IP6 was used in control experiments.

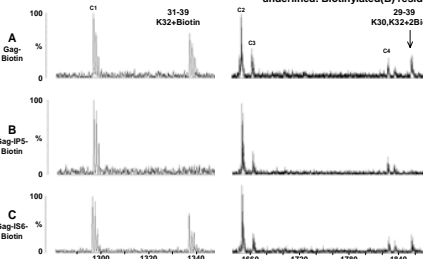


Fig. 5: MALDI spectra of tryptic digest of Gag-IP5 complex from (left) m/z 1220-1350 (right) 1650-1850. 2.5 μM of Gag was treated with (a) 0 μM of IP5 (b) 10 μM of IP5 and (c) 10 μM of IP6. The adjacent tryptic peptides were used as controls (C1, C2, C3).

The comparisons of Gag:IP5 and Gag:ssDNA interactions were made. In the MA domain, we observed protection of K30 and K32 in the Gag:ssDNA complex. Therefore, the relative affinities of IP5 and ssDNA to this site was assessed. A dose-dependent titration of the ligands with Gag was performed. Figure 6 indicates that 10 μM IP5 prevented the biotinylation of K30, while at least 40 μM ssDNA was necessary to observe the protection. The results from these experiments suggest that lower concentrations of IP5 is required compared to ssDNA in order to bind to the K30 and K32 in the MA domain. On the other hand, the residues K397, K424 and K414 located in the two different zinc binding regions of the NC domain bind exclusively to ssDNA.

MGARASVLSGGELDRWEKILRLPGGKKYKLIKHIWASRELERFAVNPGL  
LETSBGRQRLQLQPSLQTGSEELSLYNTVATLYCVHQRLEIKDTKEA  
LDKITEEQNSKKKAQQAADTGHSSQVSQNYPIVQNIQGMVQHAISFR  
TLNANVVKVVEEKAFSPEVIMFMSALSEGATPDQLNTMLNTVGGHAQMOM  
LKETINEEAEDRWHPVHAGPIAPGQMRPERGSDIAGTSTLQEQIIGMW  
TNPNPIPVGSEIYKRWIILGLNKLIVRMYSPTSILDIRGKPEFRDVFDRF  
YKTLRAEQASQEVKNMNTETLLVQNPANPDCKTILKALGPAATLEEMMTAC  
QGVGGPGHKARVLAEAMSQVNTATIMMQRGNFRNQKRMVKCFNCKGEGH  
TARNCRAPRKKKCGKCKGEGHMDCTERQANFLGRIPVSYKGRDPCNF

Fig. 4: Amino acid sequence of Gag protein. The peptides identified by MS are underlined. Biotinylated(B) residues are indicated.

The next goal was to examine the Gag-IP5:ssDNA complex. Earlier literature reports<sup>3</sup> suggested that IP5 and nucleic acid are necessary for the correct assembly of virus-like particles in vitro. We observed that lysine residues that were protected in Gag:IP5 and Gag:ssDNA complex were indeed protected in the tri-complex. Interestingly, as shown in Figure 7, we also observed protection of an additional lysine residue, K314 on the Gag protein. From earlier literature reports,<sup>2</sup> it is known that the α-helix of the CA domain, encompassing the K314, is critical for stabilizing the C-terminal dimer of the CA domain.

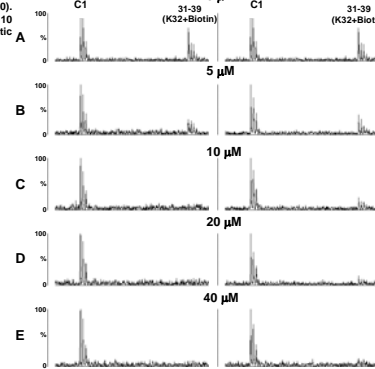


Fig. 6: MALDI Spectra comparing IP5 and ssDNA binding to 2.5 μM recombinant HIV-1 Gag. Gag:IP5 (left) and Gag:ssDNA (right) complexes were preformed and treated with NHS-biotin. The adjacent tryptic peptides were used as controls (C1).

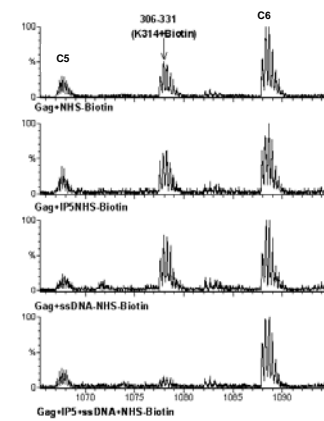


Fig. 7: ES Mass spectra (Q-ToF) demonstrating specific protection of K314 in Gag in the presence of 20 μM of IP5 and ssDNA. The concentration of Gag protein used was 2.5 μM. The adjacent tryptic peptides were used as controls (C5 and C6)

## Discussion

K30 and K32 are located in the MA domain of the Gag protein, while K314 is located in the CA domain. The observation that lower IP5 concentrations (compared to ssDNA) are required to bind to the MA domain supports literature studies implicating the MA domain association with the plasma membrane. The negatively charged phosphate groups of IP5 mimic the acidic end of the lipid groups in the plasma membrane. K397, K424 and K414 in the NC domain bind exclusively to ssDNA.

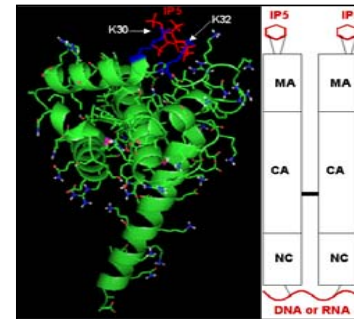


Fig. 8: A cartoon of the Gag protein summarizing the derived conclusion based on the results.

This result support the hypothesis that the NC domain is involved in interaction with the viral RNA. K314 is protected only in the presence of IP5 and ssDNA. Crystallographic and site-directed mutagenesis studies have shown that the CA domain (consisting of K314) is involved in stabilizing the C-terminal dimer of CA domain (Figure 8). Therefore, the protection of K314 observed in our studies is possibly due to protein-protein interactions established by separate Gag molecules. This conclusion is also supported by the lack of formation of virion-like particles in the absence of both IP5 and ssDNA (Refer Figure 1).<sup>4</sup>

	Gag +NHS- Biotin	Gag-IP5 +NHS- Biotin	Gag-ssDNA +NHS- Biotin	Gag-IP5-ssDNA +NHS-Biotin
K18	+	+	+	+
K26	+	+	+	+
K30	+	+	+	+
K32	+	+	+	+
K95	+	+	+	+
K103	+	+	+	+
K110	+	+	+	+
K114	+	+	+	+
K157	+	+	+	+
K272	+	+	+	+
K290	+	+	+	+
K302	+	+	+	+
K314	+	+	+	+
K335	+	+	+	+
K359	+	+	+	+
K391	+	+	+	+
K397	+	+	+	+
K416	+	+	+	+
K411	+	+	+	+
K415	+	+	+	+
K424	+	+	+	+

Table 1: (a) Table indicating lysine protection (~20 μM IP5, ssDNA) in Gag protein upon addition of IP5 and/or ssDNA.

## Conclusions

Mass spectrometric protein foot-printing was used to obtain useful structural information on Gag:IP5:DNA interactions. Lower concentrations compared of IP5 compared to ssDNA were required for MA domain binding. ssDNA binds exclusively to NC domain. K397, K424 and K414 in the NC domain bind exclusively to ssDNA. The above results serve to understand the protein-protein interactions during the assembly of the virion like particles. Analogous studies can aid in the initial rapid screening of various inhibitors that target HIV-1 assembly.

## References

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